RayBio® Human/Mouse/Rat Cyclic AMP Enzyme Immunoassay Kit

Catalog #: EIA-cAMP, EIAM-cAMP, EIAR-cAMP

User Manual
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Caution:
Extraordinarily useful information enclosed

RayBiotech
The protein array pioneer

ISO 13485 Certified

3607 Parkway Lane, Suite 100
Norcross, GA 30092
Tel: 1-888-494-8555 (Toll Free) or 770-729-2992, Fax:770-206-2393
Web: www.RayBiotech.com, Email: info@raybiotech.com

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Please read the entire manual carefully before starting your experiment

I. Introduction

Cyclic adenosine monophosphate (cAMP, cyclic AMP, or 3',5'-cyclic adenosine monophosphate) is a second messenger important in many biological processes. cAMP is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms, such as transferring into cells the effects of hormones like glucagon and adrenaline, which cannot pass through the plasma membrane. cAMP functions through three main effectors: PKA, the guanine-nucleotide-exchange factor (GEF) EPAC and cyclic-nucleotide-gated ion channels. The intracellular levels of cAMP are regulated by the balance between the activities of two enzymes adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE).

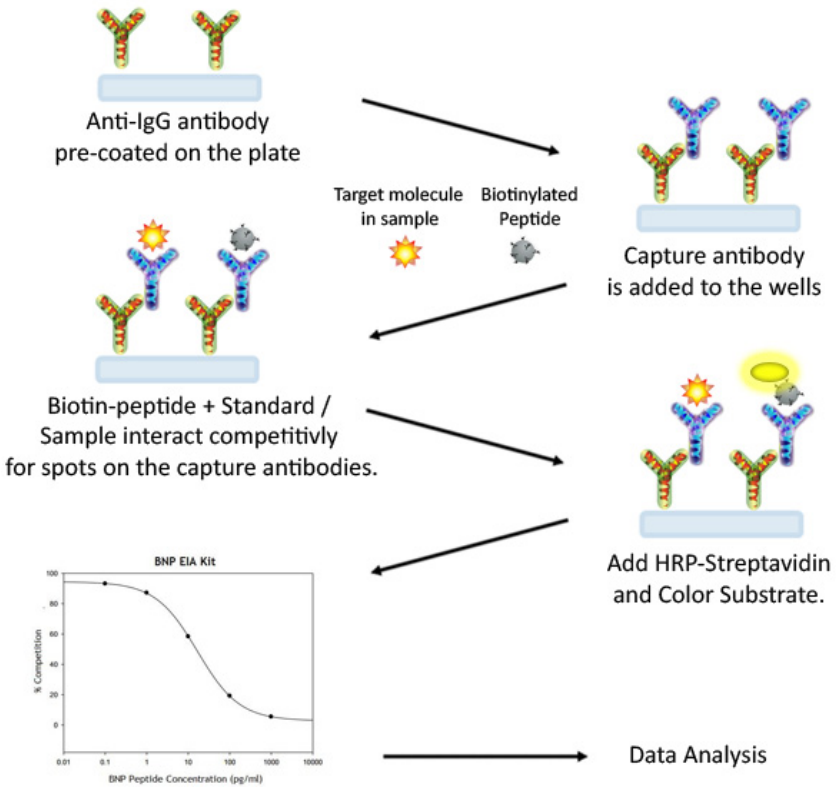
Cyclic AMP has been shown to be involved in cell growth, differentiation and general metabolism, and it is important for many biological function, especially in the cardiovascular, nervous and immune systems. The measurement of intracellular Cyclic AMP in tissues and cell cultures may help to provide a clearer understanding of the physiology and pathology of many disease states.

II. General Description

The RayBio® cAMP Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting cAMP peptide based on the competitive enzyme immunoassay principle.

In this assay, a biotinylated cAMP peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated cAMP peptide competes with endogenous (unlabeled) cAMP for binding to the anti-cAMP antibody. After a wash step, any bound biotinylated cAMP then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated cAMP peptide and inversely proportional to the amount of endogenous cAMP in the standard or samples. A standard curve of known concentration of cAMP peptide can be established and the concentration of cAMP peptide in the samples can be calculated accordingly.

III. How It Works



IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	**Size / Description**	**Storage / Stability After Preparation**
cAMP Microplate (Item A)	96 wells (12 strips x 8 wells) coated with secondary antibody.	1 month at 4°C*
Wash Buffer Concentrate (20X) (Item B)	25 ml of 20X concentrated solution.	1 month at 4°C
Standard cAMP Peptide (Item C)	2 vials of Lyophilized cAMP Peptide. 1 vial is enough to run each standard in duplicate.	Do not store and reuse
Anti-cAMP Polyclonal Antibody (Item N)	2 vials of Lyophilized anti-cAMP.	Do not store and reuse
Assay Diluent D (Item K)	15 ml of 5X concentrated buffer. Diluent for standards and samples.	1 month at 4°C
Assay Diluent B (Item E)	15 ml of 5X concentrated buffer. Diluent for anti-cAMP antibody and HRP-Streptavidin.	1 month at 4°C
Biotinylated cAMP Peptide (Item F)	2 vials of Lyophilized Biotinylated cAMP Peptide, 1 vial is enough to assay the whole plate.	Do not store and reuse
HRP-Streptavidin Concentrate (Item G)	600 µl 200X concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control (Item M)	2 vials of Lyophilized Positive Control.	Do not store and reuse
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid.	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Adjustable 1-25 ml pipettes for reagent preparation
4. 100 ml and 1 liter graduated cylinders
5. Absorbent paper
6. Distilled or deionized water
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions
9. Orbital shaker
10. Aluminum foil
11. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

A. Preparation of Plate and Anti-cAMP Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.

2. Label removable 8-well strips as appropriate for your experiment.

3. Assay Diluent D (Item K) and 5X Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.

4. Briefly centrifuge the anti-cAMP antibody vial (Item N) and reconsititute with 55 µl of 1X Assay Diluent B to prepare the antibody concentrate. Pipette up and down to mix gently.

5. The antibody concentrate should then be diluted 100-fold with 1X Assay Diluent B. This is your anti-cAMP antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

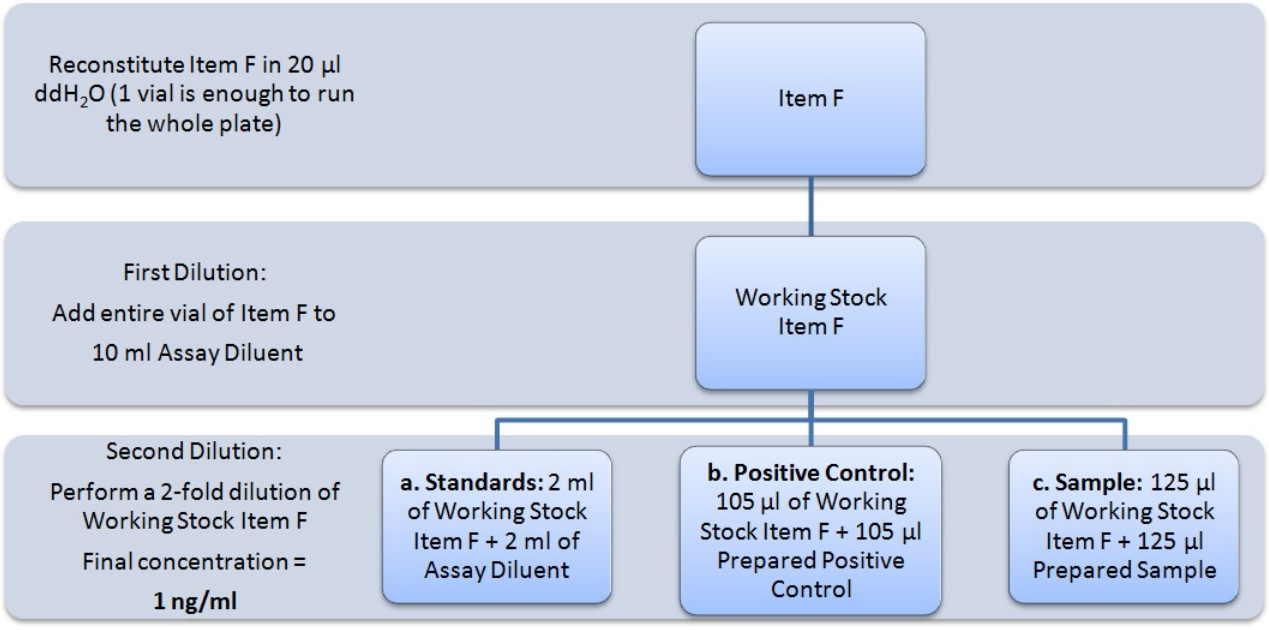
Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated cAMP (Item F)

5. Briefly centrifuge the vial of Biotinylated cAMP (Item F) and reconstitute with 20 µl of ddH_2O before use.

6. See the image below for proper preparation of Item F. Transfer the entire contents of the Item F vial into a tube containing 10 ml of 1X Assay Diluent D. This is your Working Stock of Item F. Pipette up and down to mix gently. *The final concentration of biotinylated cAMP will be **2 ng/ml***.

    a. Second Dilution of Item F for Standards: Add 2 ml of Working Stock Item F to 2 ml of 1X Assay Diluent D The final concentration of biotinylated cAMP will be **1 ng/ml**.
    b. Second Dilution of Item F for Positive Control: Add 105 µl of Working Stock Item F to 105 µl of the prepared Positive Control (Item M). (See section D for Positive Control preparation) The final concentration of biotinylated cAMP will be **1 ng/ml**.
    c. Second Dilution of Item F for samples: Add 125 µl of Working Stock Item F to 125 µl of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated cAMP will be **1 ng/ml**.

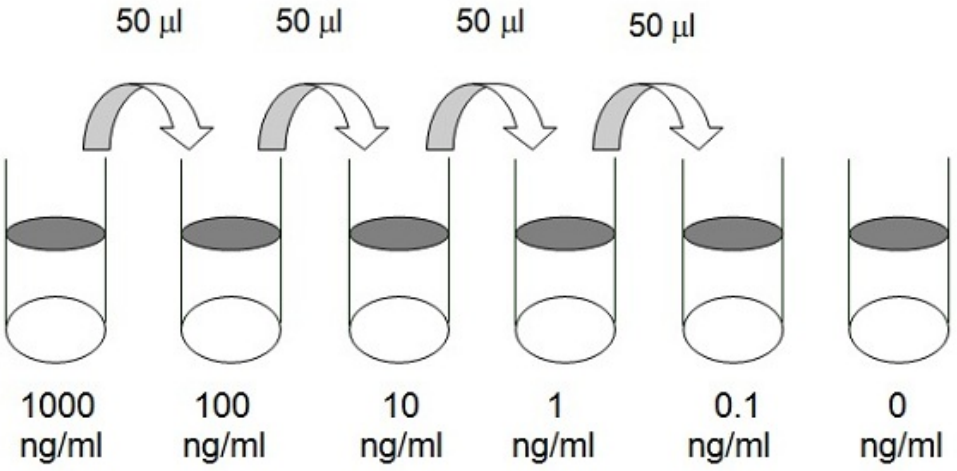


C. Preparation of Standards

7. Label 6 microtubes with the following concentrations: 1,000 pg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 0.1 ng/ml and 0 ng/ml. Pipette 450 µl of biotinylated cAMP Item F working solution (prepapred in step 6a) into each tube, except the 1,000 ng/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated cAMP is 1 ng/ml in all standards.

8. Briefly centrifuge the vial of cAMP Standard (Item C). Reconstitute with 10 µl of ddH_2O and briefly vortex if desired.



D. Positive Control Preparation

11. Briefly centrifuge the Positive Control vial (Item M) and reconstitute with 100 µl of ddH_2O.

12. Refer to step 6b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated cAMP should still be 1 ng/ml.

The Positive Control is a mouse serum sample sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated cAMP is 1 ng/ml.

E. Sample Preparation

13. If you wish to perform a 2-fold dilution of your sample, proceed to step 6c. If you wish to perform a higher dilution of your sample, dilute your sample with 1X Assay Diluent D before performing step 6c.
    EXAMPLE (to make a 4-fold dilution of sample):
    a. Dilute sample 2-fold (62.5 µl of sample + 62.5 µl of 1X Assay Diluent D).
    b. Perform step 6c (125 µl of working solution Item F + 125 µl of sample prepared above).

    The total volume is 250 µl, enough for duplicate wells on the microplate.

It is very important to make sure the final concentration of the biotinylated cAMP is **1 ng/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may contact technical support (888-494-8555; techsupport@raybiotech.com) to obtain recommended dilution factors for serum.

F. Preparation of Wash Buffer and HRP

14. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved.

15. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.

16. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. Do **not** vortex this solution as it is sensitive to oxygen.

17. Dilute the HRP-Streptavidin concentrate 200-fold with 1X Assay Diluent B.

*Note: do **not** use Assay Diluent D for HRP-Streptavidin preparation in step 17*

VIII. Assay Procedure

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.

2. Add 100 µl of Anti-cAMP Antibody (Item N) (See Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycle/sec). You may also incubate overnight at 4ºC.

3. Discard the solution and wash wells 4 times with 1X Wash Solution Buffer (200-300 µl each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of each standard (see Reagent Preparation Section C), Positive Control (see Reagent Preparation Section D) and sample (see Reagent Preparation Section E) in appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) overnight or at 4ºC.

5. Discard the solution and wash 4 times as directed in Step 3.

6. Add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation

step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking. It is recommended that incubation time should not be shorter or longer than 45 minutes.

7. Discard the solution and wash 4 times as directed in Step 3.

8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).

9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.

2. Add 100 µl anti-cAMP to each well. Incubate 1.5 hours at room temperature or overnight at 4ºC.

3. Add 100 µl standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4ºC.

4. Add 100 µl prepared Streptavidin solution. Incubate 45 minutes at room temperature.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

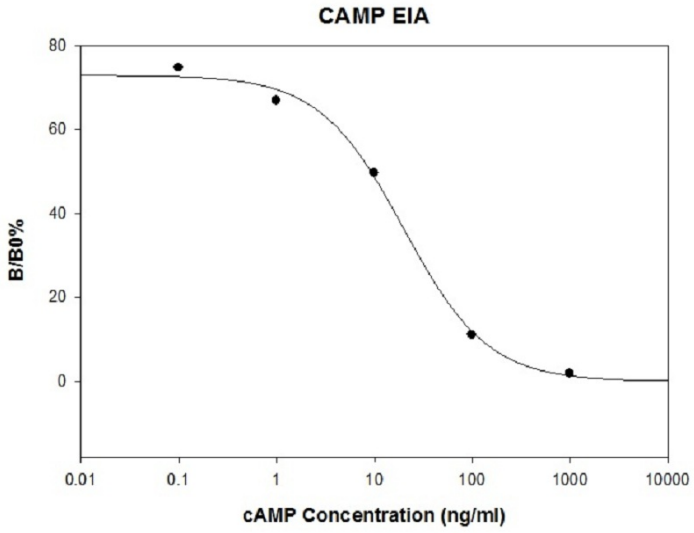
X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance = (B-blank OD)/B_0-blank OD) where
B = OD of sample or standard and
B_0 = OD of zero standard (total binding)

A. Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. Sensitivity

The minimum detectable concentrations of cAMP is 4.4 ng/ml.

C. Detection Range

0.1-1,000 ng/ml

D. Reproducibility

Intra-Assay: CV<10%
Inter-Assay: CV<15%

E. Assay Diagram

Recommended Plate Layout:

Blank	Blank	Pos Control	Pos Control	SA8	SA8	SA16	SA16	SA24	SA24	SA32	SA32
Total Binding	Total Binding	SA1	SA1	SA9	SA9	SA17	SA17	SA25	SA25	SA33	SA33
Standard1	Standard1	SA2	SA2	SA10	SA10	SA18	SA18	SA26	SA26	SA34	SA34
Standard2	Standard2	SA3	SA3	SA11	SA11	SA19	SA19	SA27	SA27	SA35	SA35
Standard3	Standard3	SA4	SA4	SA12	SA12	SA20	SA20	SA28	SA28	SA36	SA36
Standard4	Standard4	SA5	SA5	SA13	SA13	SA21	SA21	SA29	SA29	SA37	SA37
Standard5	Standard5	SA6	SA6	SA14	SA14	SA22	SA22	SA30	SA30	SA38	SA38
Standard6	Standard6	SA7	SA7	SA15	SA15	SA23	SA23	SA31	SA31	SA39	SA39

XI. Specificity

This EIA kit is designed to only detect Cyclic AMP.

XIV. Select EIA Publications

1. Plum L, Lin HV, Dutia R, Tanaka J, Aizawa KS, et al. The Obesity Susceptibility Gene Carboxypeptidase E Links FoxO1 Signaling in Hypothalamic Pro-opiomelanocortin Neurons with Regulation of Food Intake. Nature Med. 2009;15(10):1195-1201. (Ghrelin EIA, EIA-GHR-1)
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4. Revollo, J.R., et al. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. J. Biol. Chem. 2004; 279: 50754-50763.
5. Oh-I S, Shimizu H, Satoh T, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 2006; 443 (7112): 709-12.
6. Zhang J, Ren P, Avsian-Kretchmer O, Luo C, Rauch R, Klein C, Hsueh A. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 2005; 310 (5750): 996-9.
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8. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2002; 407 (6806): 908-913.9. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999; 402 (6762): 656-60.

XIII. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	• Inaccurate pipetting • Improper standard dilution	• Check pipettes • Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing
Low signal	• Improper preparation of standard and/or biotinylated antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution	• Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; assay procedure step 2 may be done overnight • Check pipettes and ensure correct preparation
Large CV	• Inaccurate pipetting • Air bubbles in wells	• Check pipettes • Remove bubbles in wells
High background	• Plate is insufficiently washed • Contaminated wash buffer	• Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	• Improper storage of the ELISA kit • Stop solution	• Follow storage recomendations in sections IV and V. Keep substrate solution protected from light. • Add stop solution to each well before reading plate

RayBio® ELISA Kits

Over 2,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

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